HPLC Separation of Peptides from Human Blood

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Abstract: The current study reviews previous reports on High Performance Liquid Chromatography (HPLC) related to biological liquids that contain biologically active peptides. Since various types of HPLC can be used to study peptides in biological liquids, including human blood, the medical, biological, and criminological applications of HPLC are investigated. Furthermore, as chromatography can separate preparative secretions and identify different peptides in human blood, the application of different HPLC methods for examining the peptides in human blood is also discussed. Finally, the influence of various factors on the mechanisms, characteristics, and results of peptide separation is considered. The outcomes of HPLC studies on the peptides in human blood can be employed in other biochemical studies, used to form peptidograms, and clinically applied for diagnosis during treatment.

Keywords: HPLC, peptide, human blood, biological activity

Introduction

Modern biochemical research is impossible without modern physico-chemical methods. Various complex biological systems have already been extensively studied by biochemists, medical researchers, and pharmacists and such systems are characterized by many components and complex chemical structures. Biological liquids are sometimes characterized without proper information regarding their nature, component structures, and quantities. The currently available physico-chemical methods used to research biochemical systems, for example NMR, UV-, IR-spectroscopy, chromatography, and mass spectrometry [1-6], require adequate methods of sample preparation [7-13]. Therefore, the use of new approaches and methods is essential for investigating chemical and biological objects. From this point of view, the use of chromatography is quite attractive. The variety of purposes related to medical and biological studies is the reason for using different chromatography systems. Chromatographic systems differ according to the computer hardware and software involved basic principles of separation, and method of detection (i.e. flame ionization detection, mass spectrometry). However, liquid chromatography is most frequently used because the analyzed objects have high molecular weights, high boiling points and their biological structures can be destroyed by high temperatures [14-20]. The results according to the different types of chromatography are influenced by many factors, including the properties of the analyzed substances, selectivity of the separation system (thermodynamic properties of the system balance), kinetics, and dynamic characteristics (flow rate of the eluent, temperature, viscosity of the eluent, size of the particle sorbents; the project and size of the column; the size of the test entered into a column). As such, these factors determine the efficiency of chromatography and the quality of the results.

The following materials have been successfully analyzed using various chromatography methods: amino acids [21], peptides [22], nucleotides and medicinal compounds [23-29], phospholipids [30,31] biological objects, the structure of proteins and nucleotides [32], diagnosis of certain conditions [33], contents of medicinal substances and their metabolites in the blood, and contents of various areas of the body [34-46]. Studying the peptides in human blood is important, because peptides are biologically active and have many functions in various physiological systems. Accordingly, the purpose of the current work was to review existing literature on chromatography focusing mainly on the separation of peptides from human blood.

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Peptides in Human Blood

The proteins and peptides in human blood are large molecules that have various functions, including catalyzing fermentation (trypsin, glutamine), transportation (hemoglobin, myoglobin, whey albumin), protection (antibodies, fibrinogen, thrombin), transferring information, and hormones (insulin, glucagons) [47]. Many different biological systems in the body have identical biochemical principles of peptide synthesis. Figure 1 shows one of the stages of peptide synthesis in humans. The fermentative destruction of proteins is finished by formation of mixes from amino acids and peptides. These substances soak in mucous membrane of the human bowels. Here some peptides collapse up to short peptides and amino acids by fermentative hydrolysis. The short peptides and amino acids go into bloods system. Thus, the mix of small peptides and amino acids, which was formed at disintegration of the proteins, contains in human blood.

Peptide synthesis is the result of the consecutive hydrolysis of a protein molecule precursor and, in humans peptides are synthesized by the endothelium. The endothelium is a specific biological tissue that produces blood vessels. In addition, peptides can also be formed as result of a hemoglobin degradation process. Some peptides from the hypothalamo of area of the brain can be found in the blood. Peptides enter internal organs through the blood circulation and then begin to regulate various functions of the organs. Problems related to the mechanisms of peptide synthesis can lead to changes in the quantity in human blood, resulting in the occurrence of various pathological processes [48-50].

The peptides synthesized in human organs and blood differ considerably according to their molecular weight and other physico-chemical characteristics [51,52]. It is very important that the quantity of peptides in the blood is controlled. Therefore, the use of chromatography is convenient and preferred.

It should be noted that a chromatographic analysis of the peptides in human blood is no different from an analysis of the peptides in other biological liquids. Hemofiltrates from humans have been used frequently in chromatographic studies of the peptides in human blood [53]. The hemofiltrate of human blood contains many proteins and peptides with various functions. These distinctions can be divided into the primary structure, molecule size, spatial configuration, charge, and other properties. Interest in studying the peptides in human blood has recently emerged due to various studies that have established that the peptides in human blood have a very high biological activity. However, relatively little research has focused on peptide formation in human blood. In existing literature, there have been many attempts to analyze the peptides in biological liquids using chromatography [54,55]. However, the peptides from human blood have not yet been reported, mainly due to their short *in situ* lifespan. It has been reported that the separation of human blood peptides by liquid chromatography and mass - spectroscopy for the purpose of creating a peptide database for human blood will facilitate the production of a screening process (cartography) for various peptides [56]. Figure 2 shows the stages necessary to form a peptidogramme for human blood. Such peptidograms are used for diagnostics purposes and treatment in medicine, and for identification in criminology (forensic medicine) [57-60]. The assembled information can be kept in computer databases and the
peptide retention time. Partition of peptide mixtures is a function of the polar-polar interaction between components of the mixture, stationary phase, and mobile phase. Peptides of the sample with stronger interaction with the stationary phase will tend to “retain”, whereas components of the sample with stronger interaction with the mobile phase will tend to moving.

The solvents used in reversed-phase liquid chromatography need to be able to easily dissolve the main groups of biologically active substances. By changing the polarity of the mobile phase, it is possible to adjust the zone distribution process of the analyzed peptides in the adsorbent layer. Therefore, adjusting the selectivity of the analysis is important. The sorbents used in reversed-phase liquid chromatography can quickly reach a balance with the various solvents, thereby allowing one analysis process to proceed to another without the need to replace the column. Reverse phase chromatography uses solvents with a wide range of properties, such as salt, acid or base, ion - pair reaction substances, and organic modifiers. The regeneration of pure solvents by distillation can easily be accomplished. Pollution from sorbents that are only in the column for a short time can easily be removed by passing another solvent through the column to remove the pollutants. Thus, the character of the peptides moving between the mobile and stationary phases is determined according to the nature of the peptides and mobile phase used. Non-polar columns with nonpolar yet slightly polar sorbents are important for analyzing peptides and modeling certain biological systems. The adsorption process in such columns occurs in a similar way as regards the interaction of the biologically active substances with the cell membranes, since adsorption occurs on a nonpolar surface from an aqueous environment.

The problem of reproducibility with a peptide analysis is associated with the remaining amount of undissolved components, plus the quality of the HPLC column also partially determines the accuracy of the analysis.

Although the mechanism of peptide separation differs according to the different characteristics and nature of the components, there is still no theory that can effectively describe the mechanism of peptide separation. Theoretical models for ideal substance separation have only recently been developed [65,66], yet the practical application of these theories is quite difficult. Thus, in most cases an empirical approach is used for peptide separation, and general recommendations and instructions are the only guides [67-69].

In zone distribution process, a solute of the peptides is introduced onto the reversed stationary phase as a narrow band. As this band moves through the chromatographic system, dispersion of the peptides molecules occurs due to lateral diffusion and hence the band becomes broader. Too much dispersion can result in overlapping bands or
peaks and incomplete separation. Therefore, in order to obtain efficient separations, the mobile phase, stationary phase and equilibrium conditions need to be carefully selected to achieve the desired separation as rapidly as is feasible but with minimum dispersion or band broadening. The rate of migration of a peptides hand through a reversed phase column depends on the distribution of molecules between the mobile phase and stationary phase. The factors which influence the distribution and hence the chromatographic retention of the peptides are composition and properties of the mobile phase, type and properties of the reversed stationary phase; the intermolecular forces between the peptides and stationary and mobile phases, temperature. An optimized chromatographic separation of the peptides is achieved by varying the mobile and stationary phase properties and operating parameters to give the required chromatographic retention of the peptides. The overall retention characteristics for each peptide are related to the kinetics and mass transfer processes, leading to retention forces.

Preserving the Native Form of a Peptide

Most often, peptides are separated using a gradient mode in the mobile phase, which requires consideration of the adsorption and physico-chemical features of the peptides. Peptide molecules, which include biological liquids, have a certain charge. The size and character of this charge is determined by the nature of the amino acids, their quantity, and pH. The structure of a peptide can then be deduced using all these factors. The structure of a peptide in an aqueous solution of a biological liquid, “the native form of a peptide,” has many names. As shown in Figure 3, the native form of a peptide is a very important factor for determining its biological activity.

Preserving the native form of a peptide during sample preparation and peptide separation is crucial for obtaining reliable results.

pH Control

Hydrolysis is a basic chemical property of peptides, yet hydrolysis destroys the chemical bonds and changes the basic structure of a peptide. Therefore, since the complete or partial destruction of a peptide by the formation of short peptides with a smaller molecular weight or α-amino acids is undesirable, it is necessary to suppress hydrolysis in order to preserve the native form of a peptide. For this purpose, good control and management of the pH of the samples and eluent is important at all stages of the experiment, thereby allowing the peptides to be separated in their natural form. pH control is also important because the pH is the main factor that determines the ion force of the mobile phase, as [H\textsubscript{2}O\textsuperscript{+}] influences the peptide structure, their change in a solution, and the conditions of the sorbent’s functional groups [70,71].

Methodology to Adjust Selectivity

Mobile Phase Composition

Yet pH control is not the sole factor in peptide separation, as the correct choice of mobile phase is also important for separating peptides. The efficiency and quality of chromatographic separation is determined by the nature and the structure of the mobile phase [72]. As a rule, graded modes of the mobile phase should be used for peptide separation. In reversed-phase chromatography, which is frequently used for peptide separation, mobile phases consisting of water and alcohol [73,74], water and acetonitrile [59,60,75,76] are used. The addition of small quantities of organic acids is also necessary in the mobile phases to suppress peptide ionization and maintain the native form.

pH Gradient Method

One special form of chromatographic separation of peptides is the use a mobile phase with a changing pH, called chromatofocusing, where a pH gradient is used to effectively increase the selectivity of the HPLC system [77-81]. The formation of a pH gradient inside a column is essence of a chromatofocusing method. Separation of samples at change of pH is based on change in ion form of divided substances. The chromatographic behavior of ions and neutral molecules is different. Such ion forms of
the substances cooperate variously with stationary phase. In this case, the increase of selectivity can be reached by change of distribution coefficients of molecular and ion forms between the mobile and stationary phases. Originally, a pH gradient was used for the preparative separation of proteins and peptides [77,82], yet has since been successfully used to both separate and identify highly molecular proteins, peptides, and amino acids.

There are two basic methods for generating a pH gradient in chromatography. The first method consists of the creation of a pH gradient outside the column. Here, a pH change is accomplished with the aid of two or more buffers in the eluent [79] using special programs or a controlled pump [79,83]. The gradient can also be created with the aid of a reaction in the eluent [87] before applying the mobile phase in the column, where the pH is changed linearly, usually from 1.0 up to 2.5 units pH. However, the creation of a pH gradient for peptide separation has various problems and requires additional equipment. Plus, after completing the process of peptide separation, a long process of regenerating the chromatographic column is then necessary [77,82,83]. As such, these factors have a negative influence on the analysis time.

In the second method, a pH gradient is placed directly into the chromatographic column. Essentially, the column is equilibrated at a certain pH by a starting solution, then the pH gradient is created in this solution by increasing the pH of the eluent that passes through the column. However, this process is unable to create a smooth pH change inside the column. This method was originally used for peptide separation in the case of many closed isoelectric points (which differ by less than 0.1 units pH). Reference [84] describes the mechanism and conditions used for a pH gradient chromatography experiment, which enables the individual peptides in biological liquids and human blood, which have complex structures, to be analyzed and identified.

**Other Chromatographic Methods**

**Size-Exclusion Chromatography**

If normal or reversed phase liquid chromatography is impossible, then other forms of chromatography may be used to analyze peptides, for example, size-exclusion chromatography (SEC), which separates materials based on their molecular weights. Molecules of peptides have a characteristic molecular structure and size. In this method, molecules are separated according to their size and penetrability through the pores of sorbents [85,86], thereby facilitating the separation of chemical substances with various molecular weights. Molecular sieve networks with effective pore size permit the entrance of molecules that are smaller than the pore size and exclude molecules that are larger than the pore size. The biggest peptides molecules experience steric hindrance in permeation inside the packing pore space and move through the column primarily around the particles with fastest possible speed. As a result the biggest peptides molecules come out of the column first and the smallest ones come out last. As such, this method is widely used to separate biopolymers, peptides, oligo- and polysaccharose, nucleic acids, and even human blood cells (lymphocytes, erythrocytes), plus application to the preparative secretion (allocation) of peptides, specific ferment, and hormones on an industrial scale is already well established [87]. This method has also been applied to detect the molecular weights and identify the sizes of peptides, while other studies [88,89] have applied size-exclusion chromatography as an alternative to in vivo studies when studying biologically active peptides. In this case, UV was used as the detection source, and the allocated fractions were separated according to the biological activity of the peptides. The biological activity of the peptides was then determined from the chromatographic peaks, which concurred with the bioanalyses results in vivo.

**Affinity Chromatography**

Affinity (biospecific) chromatography is another alternative method that has good specificity and selectivity. This newest and most selective kind of chromatography utilizes highly specific interactions between one kind of solute molecule and a second molecule covalently attached (immobilized) to the stationary phase. For example, the ferment determines the substrate, while the hormone finds the reception site, resulting in a specific complex caused by the strict conformity of the respective chemical structures [90].

Currently, affinity chromatography is used to study the interaction process of biologically active molecules with natural ligands [91,92], including the secretion, clearing, and separation of peptides. For example, the separation of peptides from human blood from an ischemic heart [93] and arteriosclerosis has already been reported [94], along with the analysis and separation of polypeptides [95]. However, affinity chromatography is more frequently used to separate peptides with a large molecular weight from human blood.

The use of affinity chromatography in the formation of certain complex chemical peptides and analysis of peptide secretion from human blood has also been reported [96,97]. Affinity chromatography is a simple, reliable, cost effective, and convenient method, and the procedures involved have already been well documented, including a complete list of the materials used, equipment, and substance concentrations, plus discussions on the advantages and disadvantages of the method.
Peptides Databases

There is a current need for databases on the information resulting from the HPLC separation of peptides, to enable researchers to quickly and accurately obtain the required pH gradient, optimum conditions for the experiment, and practical tasks involved. Modern bases are a database comprising over tens thousand peptide sequences of known molecules. Entries were compiled from published reports as well as from direct submissions of experimental data of researchers. Each entry contains the peptide sequence, its molecular specificity and, when available, experimental method, observed biological activity, and publication references. Peptides database registers natural and unnatural peptides, peptides derivatives and chemically synthesized peptides. Intermediates are also collecting. Computer access to such information will certainly save time, reduce chemical wastage, and facilitate accurate procedures [82]. Computer databases have already been created in various areas of modern chemistry. However, such databases are not frequently used, often due to the many programming languages involved, different ways of transferring the data, different types of computers used, and high price of the computer programs used [62,63,98,99]. Currently, the Internet allows easy access to information and databases, plus the network publication of scientific magazines, international conferences, and university websites all represent new methods and ways of developing worldwide databases.

Conclusion

HPLC is a powerful physico-chemical and analytical method and has already been extensively applied to biologically active peptides from human blood, including trypsin, glutamine, hemoglobin, myoglobin, albumin, fibrinogen, thrombin, insulin, and glucagons. However, further study on the metabolism dynamics of peptides, plus the quantitative and qualitative contents of peptides in biological liquids is still necessary. HPLC has an important role in the development of modern biology and medicine. Depending on the mechanism of chromatographic retention related to the composition and type of the mobile phase, various chromatographic systems can be determined. For example, it is possible to study the cleanliness and stability of many medicines, the preparative allocation of proteins and peptides, and the quantitative and qualitative determination of certain peptide substances. Identifying the peptides in human blood is essential for diagnosing various hereditary illnesses. For studies on small peptides (with a weight molecular < 20-25 thousand relative units), normal or reversed phase liquid chromatography is most often used, whereas research on large peptides and different proteins uses size-exclusion chromatography. Preparative chromatographic systems are frequently used to purify biological liquids (for example, human blood) and identify individual components. Meanwhile, the complex mechanisms involved in the interaction of biologically active substances with membranes and ferments can be clarified by affinity chromatography. Finally, affinity chromatography can be used to stimulate real biological processes for practical applications. Although most current analytical methods have certain limitations, such as complicated sample preparation procedures, a combination of different chromatographic procedures can provide alternative means for biochemical study. Accordingly, since modern chromatographic equipment can provide fast and precise results, the role of HPLC in applied research is expected to significantly increase.

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